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Curvature-induced accumulation of anisotropic membrane components and raft formation in cylindrical membrane protrusions

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Abstract

Coupling between the area density of anisotropic membrane inclusions and local membrane curvature is considered theoretically for a simple case of nearly flat bilayer membrane with thin tubular membrane protrusions. Lateral phase separation, i.e. accumulation of membrane inclusions in tubular membrane protrusions was obtained for strongly anisotropic inclusions if the radius of tubular protrusions is small enough.

In accordance with these theoretical predictions we observed persistence of long tubular membrane protrusions devoid of internal rod-like microtubular structure in cells. We suggest that the stability of the tubular membrane protrusions without the inner supporting rod-like cytoskeleton is a consequence of the accumulation of anisotropic membrane components in the bilayer membrane of these protrusions.

Based on the presented theoretical and experimental results it is suggested that previously reported concentration of prominin rafts in thin tubular membrane protrusions may be caused by a curvature-induced accumulation of small prominin–lipid complexes (inclusions) in protrusions and their coalescence into larger rafts.

Keywords: Anisotropic membrane inclusions; Tubular membrane protrusions; Curvature; Prominin; Lubrol rafts

1. Introduction

Clustering of membrane components or small complexes (inclusions) into larger domains (rafts) in highly curved spherical regions (invaginations) of cell membranes has been indicated recently (Harder and Simons, 1997; Holopainen et al., 2000). The size of membrane inclusions can be very small, comprising just a few molecules (Jacobson and Dietrich, 1999). The protein–cholesterol complexes (Thiele et al., 1999; Jacobson and Dietrich, 1999) are candidates for such small membrane inclusions. It was suggested that cholesterol binding proteins may form small protein–cholesterol membrane complexes (inclusions) which may then coalesce into larger domains (rafts) (Holthius et al., 2003) upon curvature-induced clustering in highly curved spherical parts of the budding region (Thiele et al., 1999). Clustering of inclusions into larger domains (rafts) may be promoted also by direct interaction between the inclusions (Jacobson and Dietrich, 1999).

Recently, specific raft formation has been indicated also on highly curved tubular membrane protrusions (Corbeil et al., 2001). These rafts differ from rafts in the planar regions of the plasma membrane (Weigmann et al., 1997). It was shown that at subcellular level (irrespective of the cell type), the membrane protein *prominin* is preferentially localized in microvilli and other plasma membrane protrusions rather than in the planar regions of the plasma membrane (Weigmann et al., 1997; Huttner and

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Zimmerberg, 2001). Therefore, it was suggested that prominin has an important role also in generation and stabilization of plasma membrane protrusions (Huttner and Zimmerberg, 2001). Since the primary cause of the observed predominant localization of prominin in plasma membrane protrusions is not its direct interaction with the actin-based cytoskeleton (Huttner and Zimmerberg, 2001), the mechanisms determining the retention of prominin in membrane protrusions has remained largely unknown (Huttner and Zimmerberg, 2001).

Prominin is not a constituent of the classical cholesterol–sphingolipid rafts. The so-called Lubrol rafts containing prominin are likely to be a novel type of membrane rafts (domains) that are distinct from the cholesterol-sphingolipid (Triton resistent) rafts in the planar parts of the membrane (Huttner and Zimmerberg, 2001). The redistribution of prominin after mild cholesterol depletion from the membrane protrusions indicates the importance of cholesterol (Röper et al., 2000) and other lipids as the partners (Huttner and Zimmerberg, 2001) in the formation of small prominin–lipid complexes (prominin inclusions) and their clustering into larger rafts. The short-range lipid-mediated attractive interactions between membrane inclusions (Bohinc et al., 2003) may offer a possible explanation for the nature of direct (nearest-neighbour) interactions between inclusions.

The aim of this paper is to propose the mechanism that induces the observed clustering of prominin inclusions into larger domains (rafts) on highly curved tubular membrane protrusions. In our theoretical analysis the curvature dependency of the energy of the anisotropic inclusions as well as the direct interaction between inclusions are taken into account. In experiments we show that thin tubular membrane protrusions may be stable also without the inner supporting rod-like cytoskeleton indicating the specific composition of the membrane of tubular membrane protrusions.

2. Materials and methods

2.1. Cells

The lung fibroblast of Chinese hamster (V79 cells) were grown in standard conditions with EMEM (Eagle minimal essential medium) to which 10% fetal calf serum (FCS) was added. In some experiments cells were grown for 24 h in medium containing 10% FCS without cholesterol. Before fixation all cells were treated with 2 M cytochalasin B (SIGMA) for 30 min.

2.2. Scanning microscopy

The cells were fixed in 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 3–4 h at 4 °C and in 1% osmium tetroxide in the same buffer for 1 h at 4 °C. Specimens were critical point dried, and sputter-coated with gold. The specimens were examined at 15 kV with a scanning electron microscope (Jeol JSM 84A).

2.3. Tubulin staining

The cells were simultaneously fixed and extracted with a mixture of 4% formaldehyde, microtubule stabilizing buffer (Bell and Safiejko-Mroczka, 1995) and 0.5% Triton at 37 °C for 30 min. After washing in PBS and blocking of unspecific labelling with 1% BSA, the cells were immunolabelled with monoclonal anti β-tubulin (SIGMA) over night. The FITC-labelled secondary antibodies (SIGMA) were applied for 2 h at 37 °C. After washing, the cells were mounted in vectashield or vectashield with DAPI (Vector) and examined in fluorescent microscope (Eclipse 200, Nicon).

3. Experimental results

The fibroblasts treated with cytochalasin B for 30 min usually exhibit long protrusions and a globular cell body (Fig. 1(1A)). Inside these protrusion, a parallel array of microtubules are organized in a rod-like structure

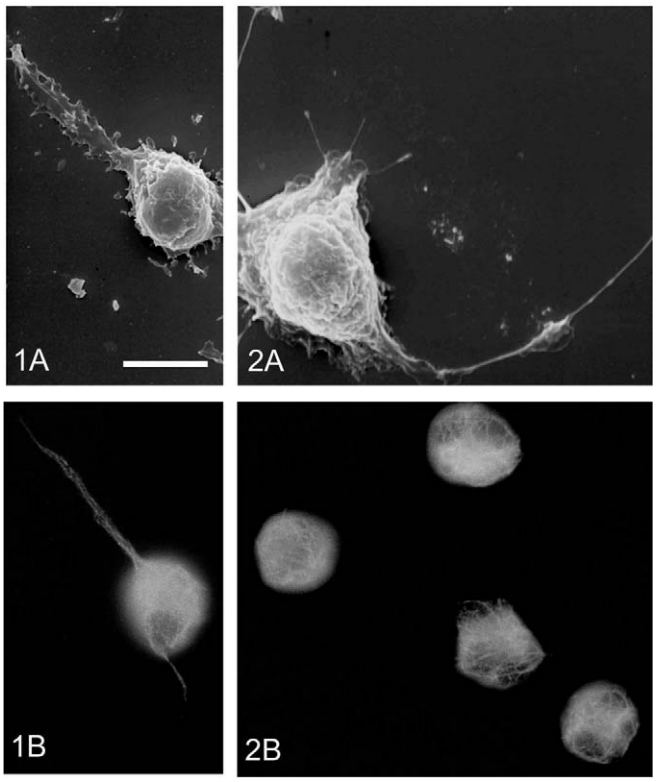

Fig. 1. In cells treated with cytochalasin B, long tubular membrane protrusions on globular cell bodies were observed (1A). Immunofluorescence labelling of tubulin shows parallel rod-like organization of microtubules in these membrane protrusions (1B). Cytochalasin treatment of cells with mild cholesterol depletion resulted in thinner and more smooth tubular membrane protrusions (2A) where the rod-like microtubular structure completely disappeared (2B). Bar = 10 μm.

(Fig. 1(1B)). On the other hand, in cells grown in a medium without cholesterol (for 24 h) the cholesterol in their membranes was moderately reduced. After treatment of these cells with cytochalasin B, the protrusions were much thinner (Fig. 1(2A)). No microspikes could be found on the protrusions (Fig. 1(2A)) in contrast to the case shown in Fig. 1(1A). In addition, in cells with reduced cholesterol, after treatment with cytochalasin B, no rod-like structures of microtubules could be found within the tubular protrusions (Fig. 1(2B)). Microtubules were concentrated only in the globular bodies of the cells close to the nuclei (Fig. 1(2B)).

It was shown that within the standard isotropic membrane elasticity models the stability of tubular membrane protrusions can not be explained without an inner supporting rod-like structure or pulling mechanical force (Miao et al., 1991, 1994; Derényi et al., 2002). On the other hand, it was shown recently that thin tubular membrane protrusions may be stabilized by anisotropic membrane components (Kralj-Iglič et al., 2005). Therefore, we suggest that the observed stability of thin tubular membrane protrusions (Fig. 1(2A)) without the inner supporting rod-like cytoskeleton (Fig. 1(2B)) may be a consequence of accumulation of anisotropic membrane components in the bilayer membrane of these protrusions.

Based on this conclusion it is suggested in our theoretical model that the reported concentration of prominin rafts in thin tubular membrane protrusions may be due to curvature-induced accumulation of small anisotropic prominin–lipid complexes (anisotropic inclusions) in protrusions and their coalescence into larger rafts.

4. Theoretical predictions

We consider a membrane with small anisotropic inclusions. The inclusions are laterally mobile. Therefore they may accumulate in regions of favourable curvature, while they are depleted from regions of unfavourable curvature (Kralj-Iglič et al., 1999; Iglič et al., 2004).

The membrane shape that would completely fit the inclusion is referred to as the shape intrinsic to the inclusion. The corresponding principal curvatures are denoted by C_{1m} and C_{2m} (Kralj-Iglič et al., 1996, 1999). In general, the local membrane shape differs from the intrinsic shape of the inclusion. This means that the principal curvatures of the actual shape differ from the principal curvatures of the intrinsic shape. The corresponding single-inclusion energy due to curvature mismatch is defined as the energy that is spent in adjusting the inclusion into the membrane and is determined by terms composed of two invariants of the mismatch tensor (Kralj-Iglič et al., 2002a,b). Terms up to the second order in the curvature tensor elements are taken into account. Upon statistical averaging over all possible orientations of the inclusion the free energy of the single inclusion can be written in the form (Kralj-Iglič et al., 1999):

$$E_i = \frac{\xi}{2}(H - H_m)^2 + \frac{\xi + \xi^*}{4}(D^2 + D_m^2) - kT \ln\left(\mathrm{I}_0\left(\frac{(\xi + \xi^*)\, D_m D}{2kT}\right)\right), \quad (1)$$

where ξ and ξ^* are the interaction constants, $H = (C_1 + C_2)/2$ and $H_m = (C_{1m} + C_{2m})/2$ are mean curvatures, $D = (C_1 - C_2)/2$ and $D_m = (C_{1m} - C_{2m})/2$ are curvature deviators, C_1 and C_2 are the two principal membrane curvatures, I_0 is modified Bessel function and kT is the thermal energy.

Within the approach of lattice statistics (Hill, 1986) we can write the normalized free energy of the inclusions $f_{in} = F_{in}/(A/a_o)$ (free energy per site) in a simple form:

$$f_{\mathrm{in}} = kT \int n \ln n \, \mathrm{d}a + kT \int (1 - n) \ln(1 - n) \, \mathrm{d}a + \frac{cw}{2} \int n^2 \, \mathrm{d}a + \int n E_i \, \mathrm{d}a, \quad (2)$$

where $n(\mathbf{r})$ is the fraction of the membrane area covered by inclusions at given position $\mathbf{r}$, A is the membrane area, a_o is the area per inclusion, w is the nearest-neighbour energy between the inclusions (Hill, 1986; Markin, 1981), c is the number of the nearest neighbours and a is the normalized (relative) area of the membrane ($a = 1$). The integration is performed over the entire (normalized) area of the membrane surface. The first two terms in Eq. (2) represent the configurational entropy (Hill, 1986; Markin, 1981). The third term describes the nearest-neighbour interaction energy between the inclusions written within the Bragg–Williams approximation (Hill, 1986).

The fraction of the membrane area covered by the inclusions $n(\mathbf{r})$ varies over the membrane surface as a function of the membrane curvature. By taking into account the conservation equation for all inclusions in the membrane: $\int n \, \mathrm{d}a = \bar{n}$, where $\bar{n}$ is the average value of n, a functional is constructed:

$$\int (f_{in} + \lambda\, n) \, \mathrm{d}a = \int L(n) \, \mathrm{d}a, \quad (3)$$

where λ is the Lagrange parameter. The variation is performed by solving the corresponding Euler equation $\partial L/\partial n = 0$ which gives the expression for the function n:

$$n = \frac{\vartheta \exp(-E_i/kT)}{1 + \vartheta \exp(-E_i/kT)} \left[1 - \frac{4w}{kT} \frac{\vartheta \exp(-E_i/kT)}{(1 + \vartheta \exp(-E_i/kT))^2}\right], \quad (4)$$

where $\vartheta = \exp(-\lambda)$. In the above expression the nonlinear terms in w are neglected. We assumed $c = 4$ for a square lattice (Hill, 1986). For attractive nearest-neighbour interactions $w < 0$. The parameter ϑ is determined from the condition $\int n \, \mathrm{d}a = \bar{n}$.

In the following we shall consider a simple model, relevant to discuss a possible physical mechanism which may explain the curvature-induced accumulation of small

anisotropic prominin–lipid complexes (i.e. anisotropic membrane inclusions) on highly curved tubular membrane protrusions. For this purpose the membrane is divided into two parts, the flat part with the relative area a_f and the fraction of the area covered by the inclusions n_f, and the highly curved tubular part of the membrane with the mean curvature $H = D = 1/2r$ (where r is the radius of tubular protrusions), the relative area a_t and the fraction of the area covered by inclusions equal to n_t. The parameter ϑ is determined numerically from the condition

$$n_f a_f + n_t a_t = \bar{n}, \tag{5}$$

where we take into account that $a_f + a_t = 1$ and n_f and n_t are given by Eq. (4).

Fig. 2 shows the fraction of the area of the tubular membrane protrusions covered by anisotropic inclusions (n_t) as the function of the tubular protrusions' radius (r) for three values of the intrinsic curvature deviator of the inclusions (D_m).

It can be seen that for small r and large D_m the fraction of the membrane area occupied by inclusions (n_t) is much larger than $\bar{n}$, while (n_f) is smaller than $\bar{n}$. This indicates the possibility of the curvature-induced accumulation of the membrane inclusions in highly curved tubular membrane regions. Due to high concentration of inclusions in the tubular protrusion the inclusions in protrusions may then coalesce into larger rafts. It can be also seen in Fig. 2 that for high enough values of the intrinsic curvature deviator of the inclusions D_m the value of n_t approaches unity indicating the possibility of the lateral phase separation of inclusions for high values of D_m and small values of r.

In the presented theoretical consideration (Fig. 2), the applied value for the interaction constant ξ is considerably larger than the corresponding value for a single isotropic lipid molecule (Kralj-Iglič et al., 2002a,b). This means that the inclusions considered in Fig. 2 can be also membrane prominin–lipid complexes. As it shown in Fig. 2 such inclusions may coalesce into larger rafts upon their curvature-induced clustering in membrane protrusions as previously observed (Huttner and Zimmerberg, 2001; Holthius et al., 2003). Our theoretical model provides an explanation for the observed curvature induced enrichment of raft markers in tubular membrane protrusions.

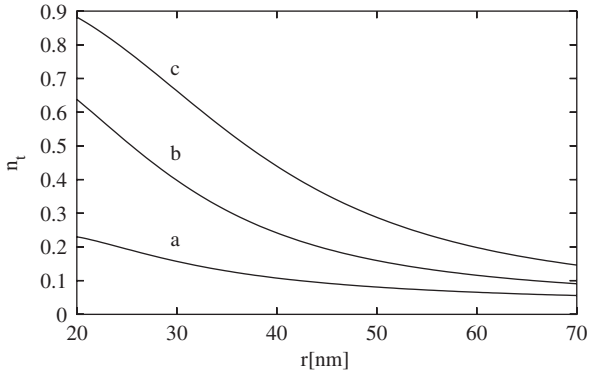

Fig. 2. The fraction the area of the membrane tubular protrusions covered by anisotropic inclusions (n_t) as a function of curvature radius of the tube (r) for three values of the intrinsic curvature deviator of the inclusions (D_m): 0.03 nm^{-1} (a), 0.04 nm^{-1} (b) and 0.05 nm^{-1} (c). The values of the other model parameters are: $\bar{n} = 0.02$, $a_2 = 0.02$, $H_m = D_m$, $w/kT = -0.12$ (Bohinc et al., 2003) and $\xi = \xi^* = 5000\,\mathrm{kT\,nm^2}$ (Iglič et al., 2004).

5. Discussion and conclusions

In this work we suggest that due to its specific molecular shape (Huttner and Zimmerberg, 2001) prominin molecules may form small anisotropic protein–lipid complexes (i.e. anisotropic membrane inclusions) which may associate into larger two-dimensional aggregates (Lubrol rafts) (Huttner and Zimmerberg, 2001; Holthius et al., 2003) upon their curvature-induced accumulation in tubular protrusions (Fig. 3). The theoretical results presented in our paper may add to a better understanding of the mechanism that induces clustering of prominin inclusions into Lubrol rafts which are considered to be a novel type of membrane rafts (microdomains) that are distinct from the cholesterol–sphingolipid (Triton resistent) rafts in the planar parts of the membrane

The coupling between the raft formation and local anisotropic membrane curvature has been recently indicated also in Golgi bodies (Sprong et al., 2001; Iglič et al.,

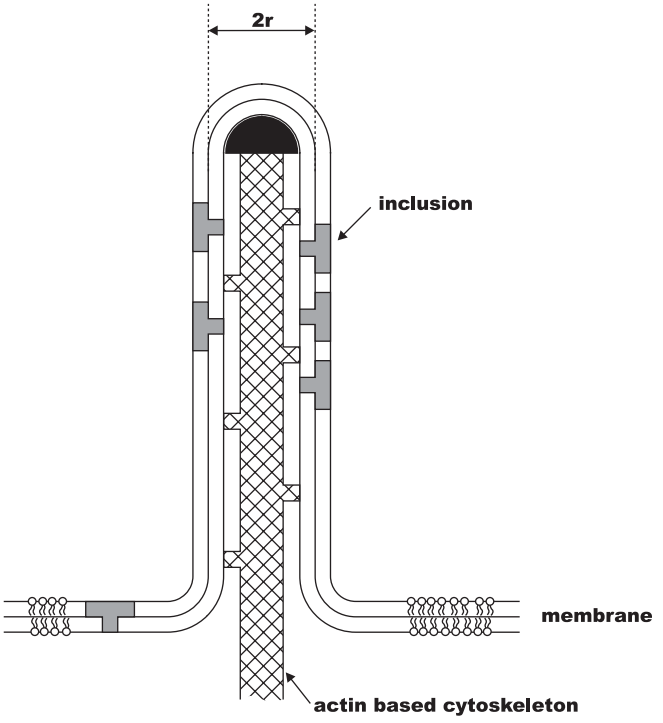

Fig. 3. Illustration of the proposed mechanism of concentration of prominin rafts in tubular membrane protrusions based on the curvature-induced lateral segregation of small prominin–lipid complexes (inclusions) with the preference for the curvature of tubular membrane protrusions where $H = D = 1/2r$.

2004) where some of the membrane components are concentrated mainly on the bulbous rims of the Golgi vesicles where the curvature deviator D is very high. Similar phenomena have been suggested also in the photoreceptor discs (Molday et al., 1987; Corbeil et al., 2001) and flattened endovesicles of erythrocyte membrane (Hägerstrand et al., 2004) indicating that the coupling between the non-homogeneous lateral distribution of the membrane rafts and the specific membrane shapes may be a general mechanism of stabilization of highly curved membrane structures (flattened disc-like vesicles, spherical buds, necks, tubular protrusions) and membrane budding process and vesiculation (Thiele et al., 1999; Holthius et al., 2003; Iglič et al., 2004).

The curvature-induced segregation and enrichment of membrane components in membrane protrusions have been discussed several times already in the past (Seifert, 1993; Kumar et al., 2001and references therein). However, all these studies were limited to short (spheroidal) membrane protrusions (buds) such as caveolae (Sens and Turner, 2004) or clathrin-coated buds (Fournier et al., 2003). In the present work, we studied for the first time the influence of the of the anisotropy of the intrinsic shape of the membrane inclusions (described by intrinsic mean curvature H_m and intrinsic curvature deviator D_m) on accumulation of the inclusions in long tubular membrane protrusions. In this study the excluded volume effect and the nearest-neighbour interactions between the inclusions are taken into account.

The predicted anisotropy-induced lateral phase separation of membrane inclusions (see Eq. (4)) may appear also without nearest-neighbour interaction term ($\sim w\int n^2\,\mathrm{d}a$) (Fig. 2). On the other hand, the nearest-neighbour interaction term alone may cause the lateral phase separation (for $w<0$) if $|w|$ is large enough. Since a specific prominin raft formation has been indicated only on highly curved tubular membrane protrusions we assume in this work that the anisotropy of prominin inclusions is the primary cause of their accumulation in thin tubular membrane protrusions while the direct interactions are secondary. In accordance, in derivation of Eq. (4) only small values of $|w|$ were assumed.

The primary cause of the physical origin of membrane protrusion is in many cases local membrane force exerted by the cytoskeleton (Boulbitch, 1998; Derényi et al., 2002). Also in these cases, the described mechanism of accumulation of anisotropic membrane inclusions in tubular membrane protrusions may offer an additional physical mechanism for stabilization of tubular membrane protrusions (Kralj-Iglič et al., 2002a,b, 2000; Yamashita et al., 2002). The observed stability of thin tubular membrane protrusions without the inner supporting rod-like skeleton (Fig. 1) is in line with the assumption that prominin inclusions (and other strongly anisotropic membrane inclusions) have an important role in generation and stabilization of plasma membrane protrusions (Huttner and Zimmerberg, 2001).

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